Award Number: W81XWH-12-1-0449

TITLE: New drugs for anemia treatment based on a new understanding of the mechanisms of stress erythropoiesis

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REPORT DATE: September 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                        Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Annual	01 September 2012 –31 August 2013

4. TITLE AND SUBTITLE

New drugs for anemia treatment based on a new understanding of the mechanisms of stress erythropoiesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0449

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Whitehead Institute for Biomedical Research
Nine Cambridge Center
Cambridge, MA 02142-1479

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We have completed and exceeded both of the goals for the first year of this project. The first goal was determining the optimum combination and concentrations of clinically-tested prolyl hydroxylase inhibitors (PHIs) and glucocorticoid receptor agonists and dissociated agonists that act on murine BFU-E progenitors in culture to increase the output of erythroid cells. We have shown that very low concentrations of both Amgen's and FibroGen's clinical- grade Prolyl Hydroxylase Inhibitors (PHIs) targeting the PHD2 enzyme synergize with very low concentrations of the corticosteroid dexamethasone in stimulating self renewal and increasing the output of both murine and, importantly, human erythroid cells. We completed the second goal - screening of a library of >2000 tested and approved therapeutic drugs and some novel molecules for additional compounds that stimulate red cell production in culture, either at the BFU-E or CFU-E level and identified 45 potential "hits." We chose 12 for detailed characterization, and showed that four non-steroid drugs and 6 steroid drugs, all approved by the FDA for other indications, stimulate expansion of murine BFU-E progenitors in culture and stimulate red cell production to the same extent as the corticosteroid dexamethasone. We have focused on one – cimetidine (Tagamet) - and showed that it and three other FDA-approved Histamine H2 receptor antagonists stimulate BFU-E self-renewal and increase the output of both murine and, importantly, human erythroid cells in culture. Following additional experiments to characterize the red cells formed in these cultures of human blood CD34+ hematopoietic stem and progenitor cells, these and other approved drugs should be poised to enter clinical trials for Diamond Blackfan anemia and other bone marrow failure disorders.

15. SUBJECT TERMS glucocorticoids, erythropoiesis, histamine receptor, histamine H2 receptor agonists, anemia, Diamond Blackfan Anemia, Prolyl Hydroxylase Inhibitors, human hematopoietic stem cells.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	30	**19b. TELEPHONE NUMBER** *(include area code)* 617–258–5216

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Introduction:

Red blood cell levels are normally tightly regulated by erythropoietin (Epo), and anemia associated with chronic renal insufficiency and malignancy can be successfully treated with recombinant Epo [1]. Epo stimulates erythropoiesis by promoting survival, proliferation, and terminal differentiation of colony-forming unit Erythroid (CFU-E) cells; over 3- 5 days each CFU-E will produce ~10- 60 mature enucleated erythroid cells [2-5]. Since normal Epo levels are very low, red cell output from CFU-E cells can be increased more than one order of magnitude by increased Epo production or by injection of recombinant Epo. However because each CFU-E cell can undergo only 3-6 terminal cell divisions under maximum Epo-stimulation, the number of CFU-E cells will eventually limit the response to Epo; the number of CFU-Es therefore determines maximum Epo-dependent erythrocyte output.

Many forms of acute and chronic anemia are not treatable with erythropoietin (Epo) because the CFU-E erythroid progenitors that respond to Epo are either too few in number or are not sensitive enough to Epo to maintain adequate red blood cell production. Treatment of Epo-resistant anemias requires a drug that acts earlier than Epo in the pathway of red cell formation, and that enhances the formation of the Epo-sensitive CFU-E red blood cell progenitors rather than mimicking Epo in stimulating the terminal proliferation and differentiation of these progenitor cells. No such therapy exists today, but research we have conducted in the past, coupled with our new work described in this report, points the way to several types of small molecule drugs that, individually or in combination, can potentially be used to expand erythroid progenitors and to treat these disorders.

In situations where the maximal Epo-dependent output is not sufficient (hemolysis, sepsis, genetic bone marrow failure diseases such as Diamond Blackfan anemia, or severe trauma), the organism attempts to increase the number of Epo-responsive cells through a mechanism known as stress erythropoiesis (SE). [6,7] Unlike steady-state erythropoiesis, which is largely regulated by Epo, SE requires other factors such as stem cell factor (SCF) and glucocorticoids (GCs) [6-8]. The therapeutic effect of the corticosteroid prednisone in patients with the red cell progenitor disorder Diamond-Blackfan Anemia (DBA) is well documented, though severe side effects limit its use [9,10].

In 2011 we reported that glucocorticoids stimulate self-renewal of early Epo-independent progenitor cells (burst-forming units erythroid or BFU-Es), over time increasing production of colony-forming units erythroid (CFU-E) erythroid progenitors from the BFU-E cells, and enhance the numbers of terminally differentiated red cells. GCs do not affect CFU-E cells or erythroblasts. In mRNA-seq experiments, we found that glucocorticoids induced expression of ~86 genes more than 2- fold in BFU-E cells. Computational analyses indicated that, of all transcription factors, binding sites for hypoxia-induced factor 1 alpha (HIF1α) were most enriched in the promoter regions of these genes, suggesting that activation of HIF1α may enhance or replace the effect of glucocorticoids on BFU-E self-renewal. Indeed, HIF1α activation by the pan-prolyl hydroxylase inhibitor (PHI) DMOG synergized with glucocorticoids and enhanced production of CFU-Es and later erythroblasts over 170-fold. PHI-induced stimulation of BFU-E progenitors thus represents a conceptually new therapeutic window for treating Epo-resistant anemia. We proposed a physiological model of stress erythropoiesis where increased levels of GCs –systemic stress hormones - and reduced oxygen – local stress - help maintain the earliest erythroid progenitors, increase CFU-E output, and at the same time stimulate terminal differentiation, thus promoting both a rapid and long-lasting increase in red blood cell production [11].

More recently we showed that the RNA- binding protein Zfp36l2 is essential for corticosteroid- induced self-renewal of early burst forming unit-erythroid (BFU-E) progenitors. Mechanistically, Zfp36l2 preferentially binds to messenger RNAs that are induced or maintained at high expression levels during terminal erythroid differentiation and negatively regulates their expression levels, including the mRNA for a key transcription factor required for erythroid differentiation. Zfp36l2 therefore functions as part of a molecular switch promoting BFU-E self-renewal and a subsequent increase in the total numbers of CFU-E progenitors and erythroid cells that are generated [12].

These studies led us to generate two hypotheses that underlie the research supported by this grant.

First, we proposed that combinations of specific prolyl hydroxylase inhibitors, those that indirectly activate the transcription factor HIF-1α, and also low concentrations of certain glucocorticoid receptor agonists or partial agonists, can significantly expand mouse bone marrow and human blood erythroid progenitors in culture. We hypothesize that this novel combination of chemical activators will stimulate the terminal proliferation and differentiation of these progenitor cells and lead to enhanced production of red blood cells without the side effects of prolonged corticosteroid therapy.

Second, we hypothesized that small molecule drugs already approved for other indications might also be able to stimulate BFU-E self-renewal and thus serve as additional therapeutic agents that could increase output of red blood cells and treat anemias untreatable by erythropoietin. Thus we proposed to use our mouse fetal liver BFU-E culture system to screen a library of ~2000 tested and approved therapeutic and other compounds for novel molecules that can stimulate red cell production in culture, either at the BFU-E or CFU-E level.

Our immediate aim is to provide new and improved treatments for young children with Diamond Blackfan Anemia and other bone marrow failure disorders. Our work will likely lead to treatments for several types of Epo resistant anemias that affect our active duty personnel as well as our veterans, including kidney dialysis patients, patients with trauma or sepsis, and possibly anemia associated with malaria.

BODY

1. Production of human red blood cells in culture

Many of our advances are due to the development in our laboratory of a cell culture system that supports synchronized expansion, terminal differentiation, and enucleation of mobilized human blood CD34+ stem and progenitor cells. This work was supported by a contract from DARPA. The detailed protocol is shown in **Figure 1;** human mobilized CD34+ blood cells were obtained from the Fred Hutchinson Cancer Center. Because these cells are from pooled patient samples and were completely anonymous they are not subject to Human Experimentation restrictions.

During the initial 4 day expansion phase the cells increased in number ~10 fold, and during the subsequent 18 day differentiation stages an additional ~1600 – 3000 fold, for a total of an approximate total 16,000 30-000 fold expansion (**Figure 2**). **Figure 3** shows the morphological changes that occur during this differentiation process. After expansion and during the first differentiation stage the cells are blast-like with large nuclei. As expected for normal erythroid differentiation, during the second and third stages the nuclei become condensed; as judged by the brown benzidine staining all of the cells become hemoglobinized. Furthermore, as shown by expression of multiple cell surface markers the erythroid cells at each developmental stage were very homogenous and thus there was very synchronous cell proliferation and differentiation (not shown). As with mouse erythroid development, an increase in CD71, the transferrin receptor, is followed by an increase in CD235 (glycophorin) in all cells, followed by enucleation. **Figure 4** shows that at the end of the culture over 56% of the cells had undergone enucleation and that all of the cells were hemoglobinized; each enucleated reticulocyte contained ~30 pg hemoglobin, similar to the amount in each human red blood cells. The enucleated reticulocytes averaged 6.7 µm in diameter, similar to that of normal human red blood cells and reticulocytes.

2. Goal 1 - Determining the optimum combination and concentrations of prolyl hydroxylase inhibitors (PHIs) and glucocorticoid receptor agonists and dissociated agonists that act on BFU-E progenitors in culture to increase the output of erythroid cells

The experiment in **Figure 5** shows that the Amgen PHI inhibitor, a specific inhibitor of the PHD2 prolyl hydroxylase, is ineffective by itself. Importantly, at very low concentrations - 0.1 nM, it synergizes with 100 nM of the corticosteroid dexamethasone in stimulating formation of <u>human </u>red cells in culture, likely by stimulating self- renewal of <u>human</u> BFU-Es. As detailed in **Figure 1** these cells were first cultured in serum- free medium supplemented with StemSpan® CC100 cytokine mix but for 5 days ("Expansion"). At day 5, 10^5 cells were

switched to culture in IMDM medium including human erythropoietin, human Stem Cell Factor (SCF), and human hIL3, with or without 100 µM DMOG (the pan- prolyl hydroxylase inhibitor used in our previous studies) or 0.1 nM Amgen PHI, and with or without Dexamethasone (Dex) as indicated. Cell numbers were counted every 2 days for 20 days.

Figure 5 shows that 1 µM dexamethasone is ineffective in stimulating human red cell formation in culture, as are 0.1 nM of the Amgen PHI or 100 µM of DMOG, (Flygare, J., V. Rayon Estrada, C. Shin, S. Gupta, and H. F. Lodish. HIF-1 alpha synergizes with glucocorticoids to induce BFU-E progenitor self-renewal Blood, 117: 3435 - 3444 (2011). Note that human CD34 cells are much less sensitive to dexamethasone stimulation than are mouse fetal liver erythroid progenitors. Importantly, a combination of 0.1 nM of the Amgen PHI with 0.1 µM dexamethasone is highly effective in stimulating production of human red blood cells. We have recently obtained similar results using the Fibrogen PHI. Importantly, note that the concentration of the Amgen PHI used is six orders of magnitude less than that of the pan-PHI inhibitor DMOG.

In very recent work, we used colony- forming assays to firmly establish that both the Amgen and Fibrogen PHIs synergize with 100 nM dexamethasone in stimulating self renewal of human BFU-E cells in culture (**Figure 6**). As in **Figure 5** these cells were first cultured in serum- free medium supplemented with StemSpan® CC100 cytokine mix for 5 days ("Expansion"). They were then transferred to fresh media containing cytokines (**Figure 1**) and cultured for 3 days with the addition of 100 nM dexamethasone without or with 1 µM of the PHIs. Colony assays were then performed to quantify the numbers of BFU-E progenitors present. As expected 1 µM Dex had a small stimulatory effect, but addition of either the Amgen or FibroGen PHI caused an almost 7- fold increase in BFU-E numbers relative to control cultures.

These results indicate that inhibition of the PHD2 prolyl hydroxylase and subsequent activation of HIF-1α by the Amgen and Fibrogen PHIs facilitates the proliferation of BFU-E erythroid progenitors in an *ex vivo* human adult CD34+ cell culture system. In work in progress, we are extending the time in culture and using the conditions detailed in **Figure 1** to see if, as we expect, the cells produced in these cultures are normal human erythroblasts that undergo enucleation to form normal human reticulocytes. Importantly, activation of HIF-1α by one or both of these PHIs might be able to synergize with very low amounts of corticosteroids to achieve a similar therapeutic effect for bone marrow failure disorders as obtained with currently- used high concentration of corticosteroids.

2. Goal 2 - Screening of a library of >2000 tested and approved therapeutic compounds for others that can stimulate red cell production in culture, either at the BFU-E or CFU-E level

This goal has been our major focus during the past, first, year of support. The compounds in this screen are a subset of the NIH's Molecular Libraries Probe Production Centers Network (MLPCN), a compound library from which we have chosen ~2000 compounds, including both FDA approved compounds and novel compounds targeting various important proteins and cell processes. We tested the capacity of each of these compounds to support BFU-E expansion using a high-throughput 384 well-plate platform. **Figure 7** shows the outline of the screen. We first tried image- based automatic cell counting, but that turned out to be difficult to automate and was unreliable. We were successful using the CellTiter-Glo®Luminescent Cell Viability Assay, which counts cell numbers based on measurements of cellular ATP levels. The output of the assay is luminescence that is automatically read and tabulated. After much preliminary experimentation we decided to use 40 cells per well. This is the maximum number of cells that can grow normally in each well, and is the number that gives a smaller standard deviation than seeding with 10 to 30 cells per well. In the final screening format we found the maximum effect of the control corticosteroid dexamethasone was at Day 6 of culture – a reproducible 3- to- 4 fold stimulation (**Figure 8**), and this was used for the actual high throughput screening.

Figure 9 shows an example of the raw data from this screen. Each plate was analyzed twice. Each plate contained ~18 wells that had no additions (yellow squares) as negative controls and a similar number that contained 100 µM Dexamethasone (green diamonds) as positive controls. The average of the number of cells (measured by luminescence) in all of the control wells on each plate was set as 1.0. As expected, virtually all of the control wells exhibited less than a 2.5 – fold increase over this average value on both replicas of the assay

(**Figure 9**) indicating that the negative controls were bunched tightly together. In contrast, all of the wells containing Dexamethasone had more than a 2.5 – fold increase in cell number over the average of the negative controls and almost all were positive on both replicas of the assay. Potential "hits" were defined as those that scored above 2.5 on both replicas (red squares).

This screen identified 45 potential "hits." We chose 23 for detailed characterization, mainly on the basis that they were FDA- approved drugs used therapeutically for other indications or that they had been determined to be safe in humans and had a known mechanism of action. As exemplified by **Table 1** and **Figure 10,** we rescreened these for their ability to stimulate proliferation of purified murine fetal liver BFU-E cells in culture by counting the cells every day. The 11 "hits" analyzed in this figure and table include bumetanide, an inhibitor of carbonic anhydrase; oxyphenonium bromide, an antagonist of the muscarinic acetylcholine receptor; cimetidine, a histamine H2--- receptor antagonist; and sulfadimethoxine, a low--- dosage, rapidly absorbed, long--- acting sulfonamide. Also included were several steroid- like drugs used for other clinical indications including medroxyprogesterone, a major component of the oral contraceptive pill. **Figure 10** is a graph of the same data as in **Table 1** plotted as fold expansion; drug numbers correspond to those listed in **Table 1** and shows that all but one of these compounds indeed increased the output of erythroid cells in culture. The compounds producing the greatest output of erythroid cells were hydrocortisone, other corticosteroids, and the estrogen receptor agonist 1,3,5(10)-estratrien-3-ol-17-one 3-sulfate potassium. Importantly, all of the non-steroidal drugs except sulfadimethoxine supported significant expansion of BFU-E cells relative to the negative control cultures.

Of the 23 "hits" selected 12 were positive during the rescreening. We decided to focus on non-steroidal compounds and selected four for more detailed analysis. Their known functions and their approved uses are:
1. Bumetanide. An inhibitor of carbonic anhydrase, bumetanide is a loop diuretic in the sulfamyl family used to treat heart failure.
2. Cimetidine (Tagamet) is a histamine H2-receptor antagonist that inhibits stomach acid production. It is mainly used as an over-the-counter drug to treat heartburn and peptic ulcers.
3. Sulfadimethoxine (Albon) is a low-dosage, rapidly absorbed, long-acting sulfonamide, used in treatment of a wide range of bacterial infections
4. Propylthiouracil is a thiouracil-derived drug used to treat hyperthyroidism by decreasing the amount of thyroid hormone produced by the thyroid gland.

Figures 11 – 14 show the structures of these molecules and some of the data supporting the conclusion that they stimulate mouse BFU-E self renewal and over time expand the number of red blood cells produced from each BFU-E as well as does dexamethasone, our "control" steroid drug (graphs on top right). In the absence of any drug each BFU-E generates ~200 red cells. Addition of bumetanide at 20µM and 200 µM, sulfadimethoxine at 200 µM, propylthiouracil at 20µM and 200 µM, and cimetidine at 20µM and 200 µM, triggered an additional ~10- fold increase in the number of red cells produced, similar to treatment with 1 µM dexamethasone.

Further, addition of these drugs had no effect on the number of red cells produced in culture from each later Epo- dependent CFU-E progenitor (graphs on lower right). Third, and supporting the notion that each of these drugs enhances BFU-E self-renewal, is the demonstration that, at Day 5 of culture, terminal erythroid differentiation, monitored by the number of Ter-119+ cells in the culture, is markedly reduced by addition of three of the four drugs (graphs on lower left).

We have conducted similar experiments using 20 µM concentrations of six steroid derivatives:
1. Medroxyprogesterone, a steroidal progestin drug that acts as an agonist of the progesterone, androgen, and glucocorticoid receptors. It is not marketed for use in humans
2. Hydrocortisone hemisuccinate, a corticosteroid used in creams for the treatment of various skin disorders
3. Beclomethasone is used for the prophylaxis of asthma. As a nasal spray (Beconase, Alanase, Vancenase) it is used for treatment of hay fever and related allergies

4. Fluocinolone acetonide, a corticosteroid primarily used to reduce skin inflammation
5. Triamcinolone acetonide, a synthetic corticosteroid used topically to treat various skin conditions, and in nasal spray form to treat hay fever and related allergies
6. Flumethasone, a corticosteroid that is used in form of creams for the treatment of various skin disorders

As judged by experiments similar to those in **Figures 11 – 14**, each of these stimulated BFU-E self-renewal and triggered an increase in the number of red cells produced from each BFU-E cell ~10 fold. Because each of these steroids is likely to stimulate red cell formation by binding to and activating the glucocorticoid receptor we are placing a lower priority on analyzing in detail the action of these steroids on expansion of human CD34+ cells in culture. For the present we are focusing on the non-steroidal drugs analyzed in **Figures 11- 14**.

The above results with cimetidine were extremely promising. We hypothesized that if, as claimed, cimetidine is acting as expected as a pure H2 receptor antagonist, then other FDA- approved H2 receptor antagonists, not included in the original drug screen, should also stimulate expansion of BFU-E progenitors. And given our success in showing that two approved inhibitors of the PHD2 prolyl hydroxylase stimulated expansion of human CD34+ cells in culture, generating increased numbers of red blood cells (**Figures 5 and 6)** we decided to test cimetidine and three other histamine H2 receptor antagonists directly in erythroid cultures of human CD34+ cells (**Figures 15 – 19**.) The results were dramatically encouraging.

The four drugs tested, each with very different molecular structures, were:

1. **Figure 15** Cimetidine (Tagamet), a histamine H2-receptor antagonist that inhibits stomach acid production and that is mainly used as an over-the-counter drug to treat heartburn and peptic ulcers.
2. **Figure 16** Nizatidine, a histamine H_2-receptor antagonist that inhibits stomach acid production. It is commonly used in the treatment of peptic ulcer disease (PUD) and gastroesophageal reflux disease (GERD). It was developed by Eli Lilly and is marketed under the brand names Tazac and Axid.
3. **Figure 17** Famotidine, another H2 receptor antagonist, is marketed by Johnson & Johnson/Merck under the trade names Pepcidine and Pepcid and by Astellas under the trade name Gaster.
4. **Figure 18** Ranitidine, trade name Zantac, is a histamine H_2-receptor antagonist that is commonly used in treatment of PUD and GERD. Ranitidine is also used for the treatment of skin conditions such as hives.

The experiments were performed similar to those detailed in **Figure 5**. In brief, CD34+ blood cells were first cultured in serum- free medium supplemented with StemSpan® CC100 cytokine mix for 6 days ("Expansion"). At day 6, 10^5 cells were switched to culture in StemSpan SFEM II Medium containing IL-3, SCF, and Epo, together with the indicated drugs. **Panels a** of **Figures 15 -18** show the total number of cells generated from one plated CD34+ cell; note that the figure plots cell numbers only after Day 12 of culture since all of the cultures with or without drugs did not differ significantly in expansion before that time. Also note that the vertical axis is plotted on a log_{10} scale.

As before, addition of 0.4 µM dexamethasone (Dex) generated a mild ~5- fold increase in production of human red cells. Addition only of any of the H2 receptor antagonists at either 20µM or 200 µM had minimal effects on red cell production. Importantly, several combinations of these compounds with 0.4 µM Dex yielded an additional 2 – 8 fold increase in red cell formation over that observed in 0.4 µM Dex alone. The effective combinations included cimetidine at 20 and 200 µM, nizatidine at 20 and 200 µM, famotidine at 20 µM, and ranitidine at 20 µM.

While we have yet to carry out BFU-E colony assays on these cultures to directly establish that these combinations of drugs stimulate BFU-E self-renewal, the experiments in **Panels b** of **Figures 15 – 18** suggest that this is the case. Plotted is the percentage of cells in the population expressing the cell surface marker of terminal red cell differentiation, CD235a, or glycophorin. In cultures lacking any drug, or containing only a histamine H2 receptor antagonist, ~70% of the cells in the culture express glycophorin by Day 15; this means that most of the cells in the culture have undergone terminal differentiation from the CFU-E stage. In contrast, all of the cultures containing 0.4 µM Dex, with or without a histamine H2 receptor antagonist, are markedly delayed in the appearance of cell surface CD235. Thus these cells are less far along in differentiation that in

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cultures without Dex or just containing a H2 antagonist, consistent with the hypothesis that they remain in a less differentiated BFU-E state.

Clearly more work needs to be done to establish that the erythroid progenitors and reticulocytes formed in the cultures with Dex together with H2 histamine receptor antagonists are normal, as assayed by hemoglobin content, size, percentage enucleation, and the many protein and mRNA markers we routinely employ to establish the quality of red cells produced in culture. Nonetheless we are very optimistic that one or more of these commercially available and safe histamine H2 receptor antagonists will be useful in treatment of Diamond Blackfan anemia and other bone marrow failure disorders.

KEY RESEARCH ACCOMPLISHMENTS:

• We demonstrated that very low concentrations of both Amgen's and FibroGen's clinical- grade Prolyl Hydroxylase Inhibitors (PHIs) targeting the PHD2 enzyme synergize with very low concentrations of the corticosteroid dexamethasone in stimulating self renewal and increasing the output of both murine and, importantly, human erythroid cells.

• We completed the screening of a library of >2000 tested and approved therapeutic compounds for others that can stimulate red cell production in culture, either at the BFU-E or CFU-E level and identified many potential "hits."

• We chose 12 "hits" for detailed characterization, and showed that four non-steroid drugs, all approved by the FDA for other indications, stimulate expansion of murine BFU-E progenitors in culture and stimulate red cell production to the same extent as the corticosteroid dexamethasone. These drugs are bumetanide, a loop diuretic in the sulfamyl family used to treat heart failure; cimetidine, (Tagamet), a histamine H2-receptor antagonist that inhibits stomach acid production and is used to treat heartburn and peptic ulcers; sulfadimethoxine, (Albon), sulfonamid, used in treatment of a wide range of bacterial infections; and propylthiouracil, a thiouracil-derived drug used to treat hyperthyroidism

• We also identified 6 steroid drugs that are approved by the FDA for other indications that stimulate expansion of murine BFU-E progenitors in culture and production of red cells to the same extent as the corticosteroid dexamethasone. These are: medroxyprogesterone, a steroidal progestin drug that is not marketed for use in humans; hydrocortisone hemisuccinate, a corticosteroid used in creams for the treatment of various skin disorders; beclomethasone, used for the prophylaxis of asthma and for treatment of hay fever and related allergies; fluocinolone acetonide, a corticosteroid primarily used to reduce skin inflammation; triamcinolone acetonide, a synthetic corticosteroid used mainly to treat hay fever and related allergies; and flumethasone, a corticosteroid used in form of creams for the treatment of various skin disorders.

• We focused on cimetidine (Tagamet) - and showed that it and three other FDA- approved Histamine H2 receptor antagonists stimulate murine BFU-E self-renewal and increase the output of murine erythroid cells in culture. Importantly, these four drugs – cimetidine, nizatidine, famotidine, and ranitidine – all synergize with low concentrations of dexamethasone to stimulate ~5 fold production of red blood cells in cultures of human blood CD34+ stem and progenitor cells. Following additional experiments on human cells in culture these four drugs should be poised to enter clinical trials for Diamond Blackfan anemia and other bone marrow failure disorders.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:
- **manuscripts, abstracts, presentations**. The first public presentation of this work will likely be at the 13th Diamond Blackfan Anemia International Consensus Conference in Atlanta March 8-10, 2014. By the beginning of 2014 we hope to submit for publication two manuscripts. One will describe the human CD34+ culture system and the ability of PHIs to synergize with corticosteroids to stimulate red cell production. The other will describe the screen and focus on the Histamine H2 receptor antagonists.
- **licenses applied for and/or issued.** None yet but we plan to apply for use patents for many of the molecules we identified that stimulate red cell formation.
- **degrees obtained that are supported by this award** On August 26 Lingbo Zhang, the developer of the screen, successfully defended his PhD thesis from the National University of Singapore as part of the Singapore MIT Alliance.
- **development of cell lines, tissue or serum repositories** None anticipated

- **informics such as databases and animal models, etc.** All of our data sets resulting from the high throughput screen and further analysis of candidate drugs will be made available to the public upon publication of our projected manuscripts. All of the molecules we have tested are commercially available.
- **funding applied for based on work supported by this award** None
- **employment or research opportunities applied for and/or received based on experience/training supported by this award**. This award led to a substantial part of Lingbo's PhD thesis research, and also led to the training in robotic screening of his technician Lina Prak.

CONCLUSION: Our cell culture assays have identified several drugs approved by the FDA for other indications that could be repurposed as potential treatments for bone marrow failure disorders such as Diamond Blackfan anemia, kidney dialysis patients, patients with trauma or sepsis, and possibly anemia associated with malaria. There are currently no acceptable therapies for these disorders. We need to characterize the red cells produced in these cultures to confirm that they are normal, and to test them in animal models of these human diseases. We do believe that several of these drugs could enter clinical trials for these diseases within a year or two since their safety in humans has already been validated extensively.

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12   Zhang, L. *et al.* ZFP36L2 is required for self-renewal of early burst-forming unit erythroid progenitors. *Nature* **499**, 92-96, doi:10.1038/nature12215 (2013).

SUPPORTING DATA
TABLE 1

<mark>Name 20130129</mark>	Tube #	Day 0	Day1	Day 2	Day 3	Day 6	Day 7	Day8	Day 9
No Dexamethasone	-	5,000	15,926	136,667	213,889	177,778	159,259	111,111	122,222
Dexamethasone	+	5,000	18,889	124,815	295,000	2,596,296	2,703,704	2,703,704	1,851,852
BUMETANIDE	1	5,000	30,000	176,667	256,111	248,148	296,296	262,963	329,630
OXYPHENONIUM BROMIDE	2	5,000	29,259	142,963	278,889	255,556	377,778	518,519	537,037
1,3,5(10)-ESTRATRIEN-3-OL-17-ONE 3-SULFATE POTASSIUM	3	5,000	24,444	181,481	446,667	829,630	1,318,519	1,885,185	1,800,000
11BETA,17ALPHA,21-TRIHYDROXY-4-PREGNENE-3,20-DIONE 21-CAPRYLATE	4	5,000	9,259	19,259	39,444	774,074	1,814,815	2,911,111	3,381,481
21-ACETOXYPREGNENOLONE	5	5,000	24,444	143,704	276,111	607,407	703,704	870,370	955,556
HYDROCORTISONE HEMISUCCINATE	6	5,000	21,852	157,037	457,222	6,281,481	10,148,148	15,111,111	18,814,815
cimetidine	8	5,000	26,296	103,333	260,556	322,222	407,407	525,926	648,148
sulfadimethoxine	9	5,000	22,593	145,926	235,000	270,370	200,000	200,000	262,963
MEDROXYPROGESTERONE	10	5,000	16,296	122,963	353,889	2,851,852	3,037,037	6,407,407	6,629,630
FLUOROMETHOLONE;426-13-1	11	5,000	13,333	87,407	414,444	3,629,630	3,000,000	1,925,926	888,889

Figure 1. Culture of mobilized human peripheral CD34+ blood cells

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

\<Expansion\> (4 days)
- StemSpan SFEM medium
- 1X CC100 cytokine mix (Flt-3 ligand, SCF, IL-3, IL-6)
- 2% Pen/Strep

\<Differentiation I\> (5 days)
- IMDM
- 15% FBS
- 2mM glutamine
- 1% BSA
- 2% Pen/Strep
- holo human transferrin: 500 µg/mL
- rh insulin: 10 µg/mL
- Dexamethasone: 2 µM
- β-estradiol: 1 µM
- IL-3: 5 ng/mL
- SCF 100 ng/mL
- Epo 6U

\<Differentiation II\> (4 days)
- IMDM
- 15% FBS
- 2mM glutamine
- 1% BSA
- 2% Pen/Strep
- holo human transferrin: 500 µg/mL
- rh insulin: 10 µg/mL
- SCF 50 ng/mL
- Epo 6U

\<Differentiation III\> (9 days)
- IMDM
- 15% FBS
- 2mM glutamine
- 1% BSA
- 2% Pen/Strep
- holo human transferrin: 500 µg/mL
- rh insulin: 10 µg/mL
- Epo 2U

★ Fibronectin plates

- **Cell source: human G-CSF mobilized peripheral blood (Fred Hutchinson)**

Figure 2. CD34+ cells proliferate for 22 days
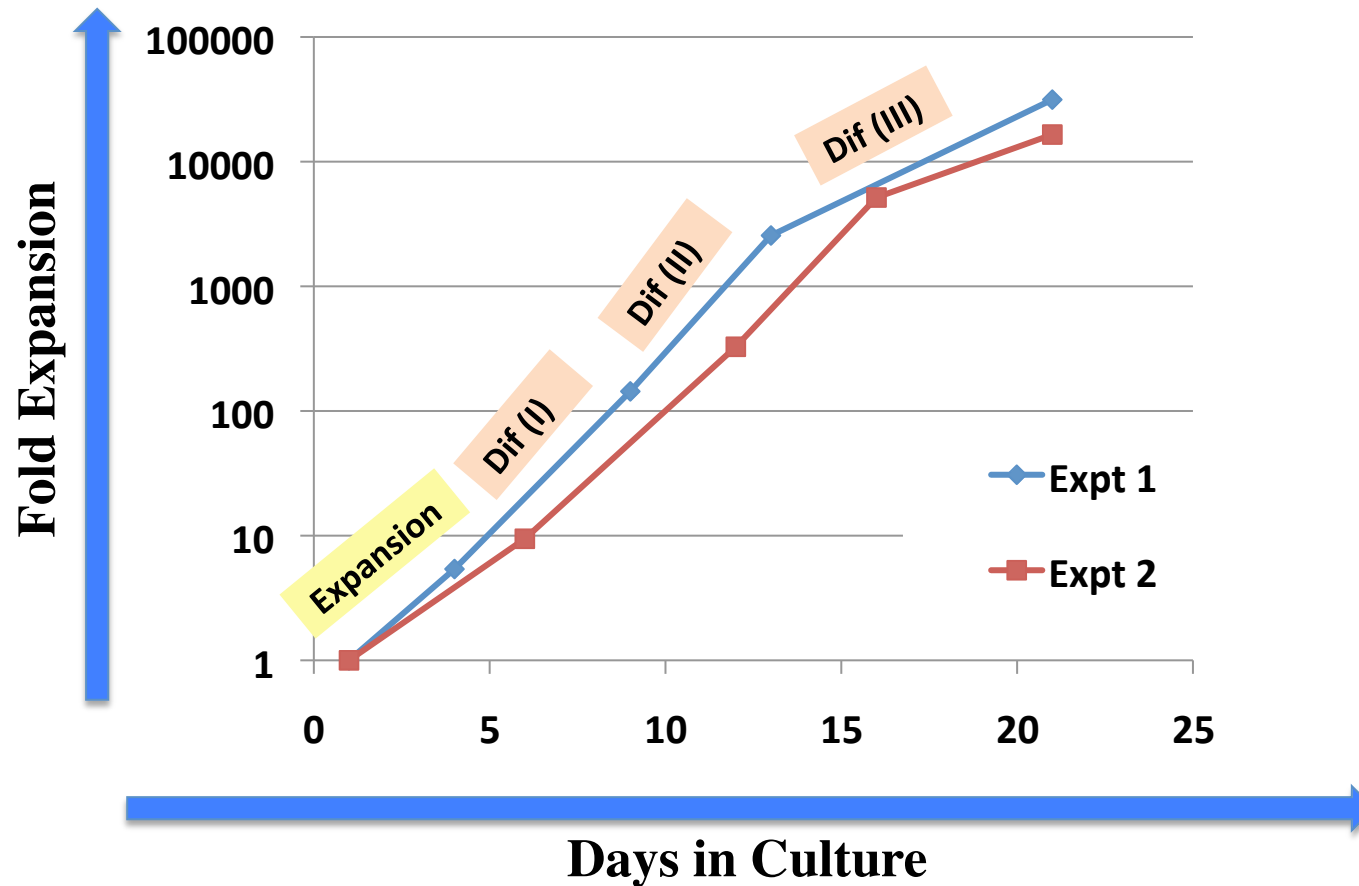
Plotted are the fold expansion of the CD34+ cells in the culture system detailed in Figure 1; two experiments are depicted
• Expt 1 (21 days): 31448.5-fold expansion (14.9- fold doubling)
• Expt 2 (21 days): 16499.9-fold expansion (14- fold doubling)

Figure 3. Cell Morphology changes during terminal differentiation of CD34+ human blood stem/ progenitor cells
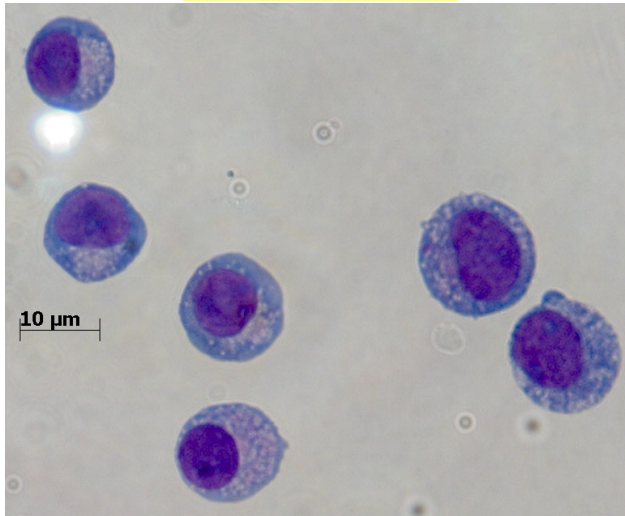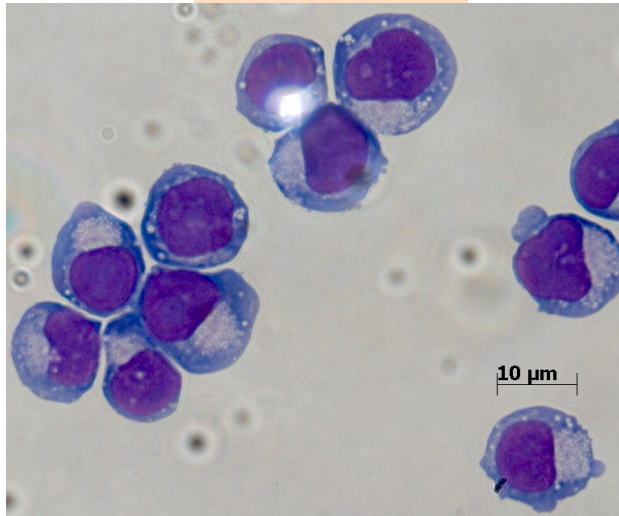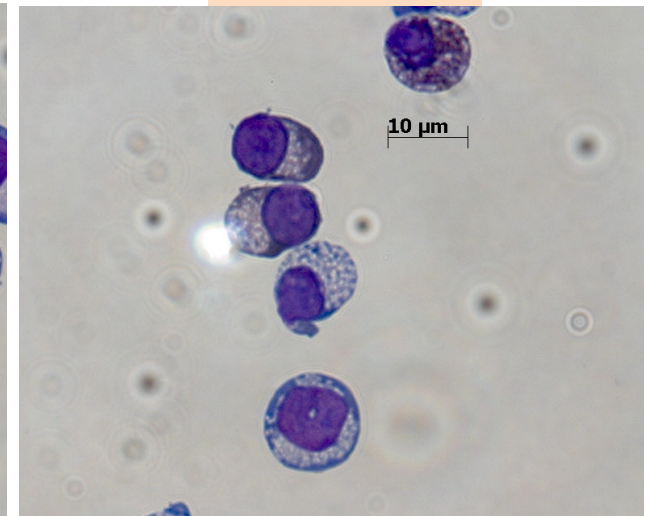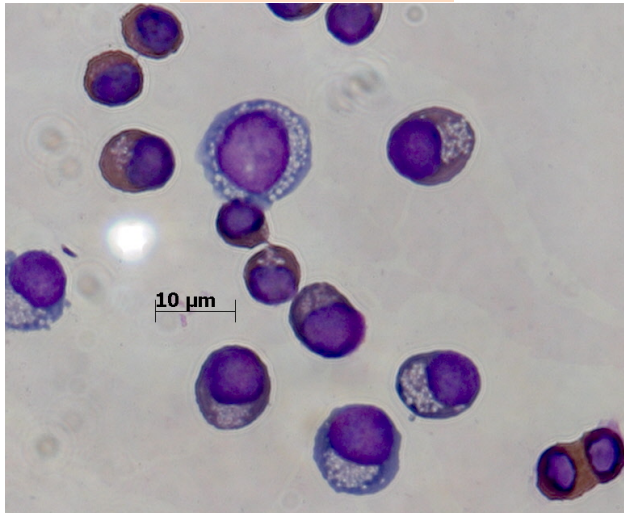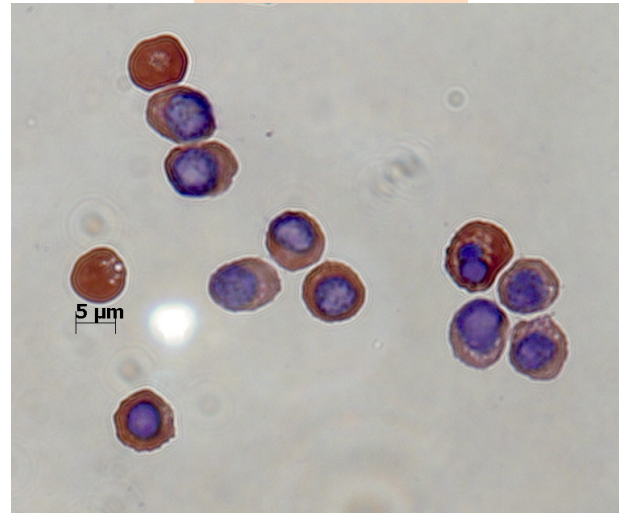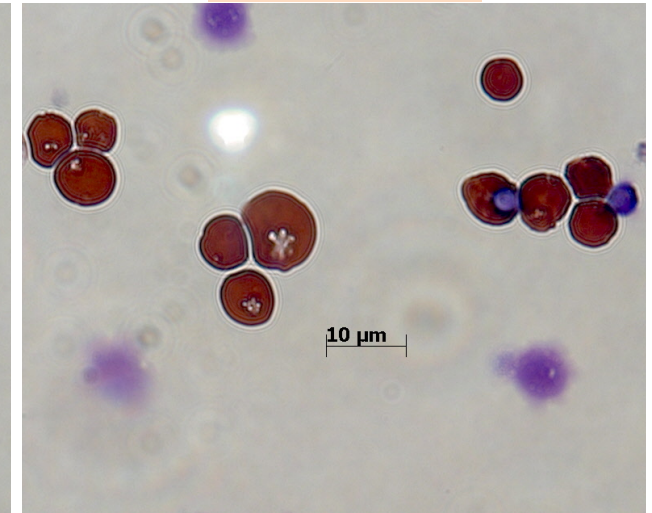
Benzidine-Giemsa staining of cells cultured as detailed in Figure 1; bar = 10μm)

Figure 4. By Day 22 of culture over 55% of the cells undergo enucleation and the size of the reticulocytes formed, – 6.7 μm diameter, is identical to that of normal human red blood cells

$$\%\text{Enucleation} = 42.6/(42.6 + 32.8) = 56.5\%$$
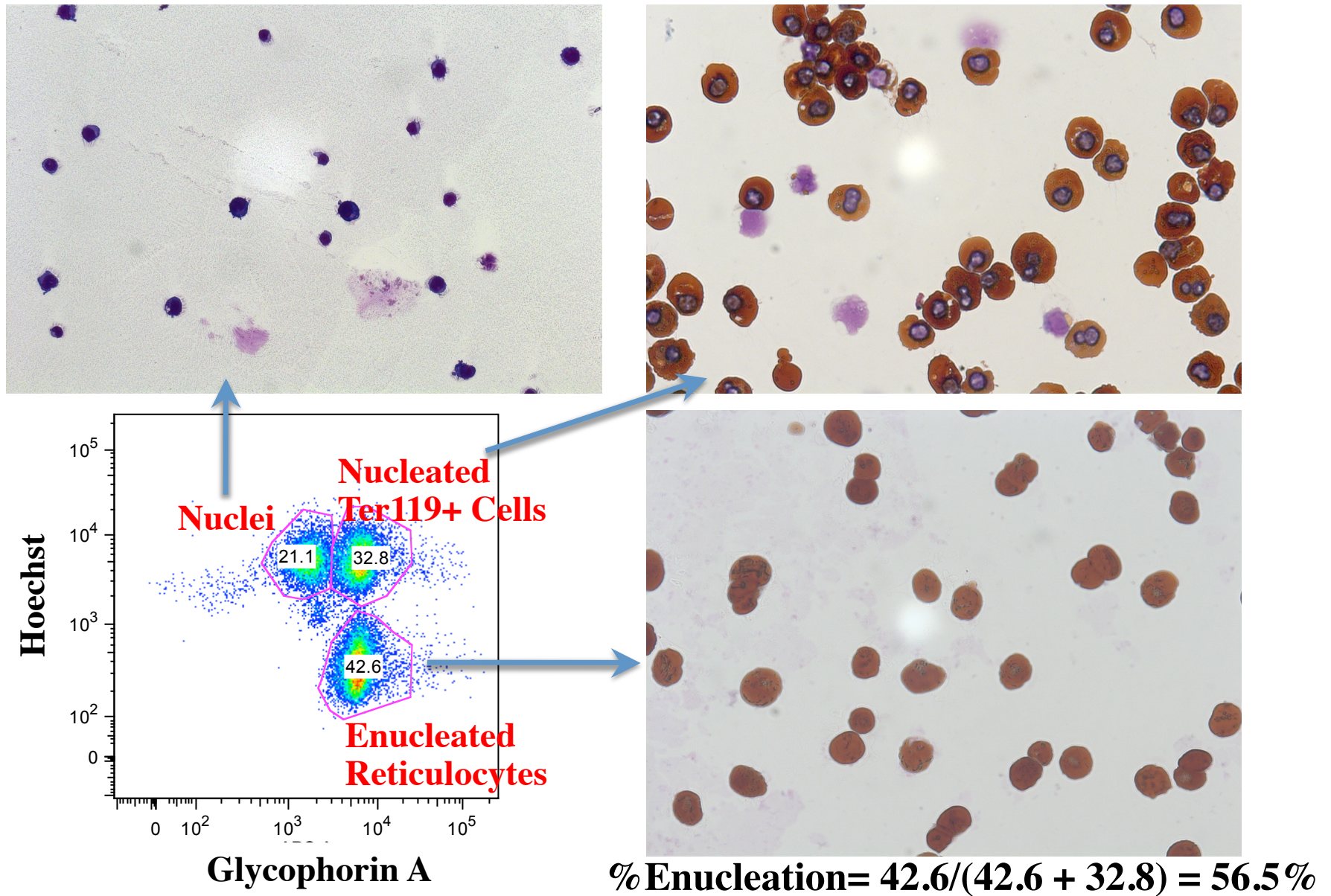
Figure 5. The Amgen PHI synergizes with Dexamethasone to stimulate self-renewal of human BFU-E cells

Legend:
- ● 1 μM Dex
- ● Untreated
- ● 0.1nM PHI
- ● 0.1nM AM-PHI + 100nM Dex
- ● 100 uM DMOG
- ● 100uM DMOG + 100nM Dex

The cell culture conditions are detailed in the text; cells were counted daily using a hemocytometer.
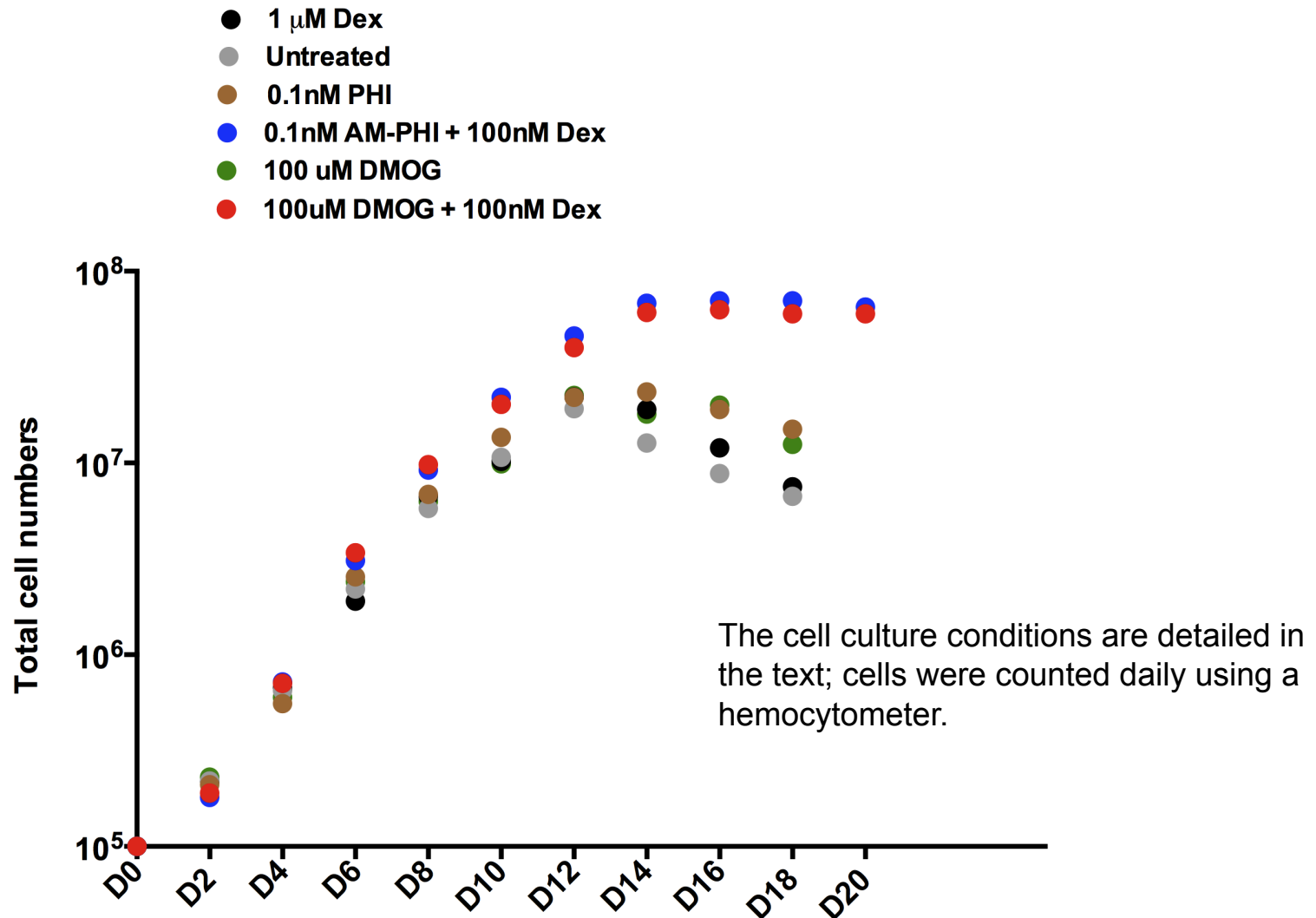
Figure 6. Both the Amgen PHI (PHI-AM) and the Fibrogen PHI (PI-FB) synergize with dexamethasone in promoting self- renewal of human BFU-E progenitors

The cell culture conditions are detailed in the text; after 3 days of culture in the presence of the indicated compounds BFU-E cells were quantified using a standard methylcellulose assay. AM= Amgen; FB = FibroGen.

Figure 7. Scheme for screening a library of ~2000 small molecules for those that can stimulate red cell production from purified mouse fetal liver BFU-E progenitors

Chemical compound library

Culture FACS sorted BFU-Es into 384 well plate

Culture for 8 days

Plate reader (Luminometer)

Counting cell number and identify positive compounds

Cell number
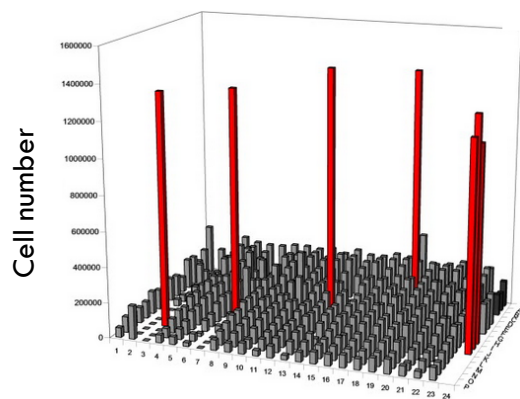
Figure 8. The final screening format: 40 sorted BFU-E cells in each well with (+) and without (-) dexamethasone at Day 6 of culture.
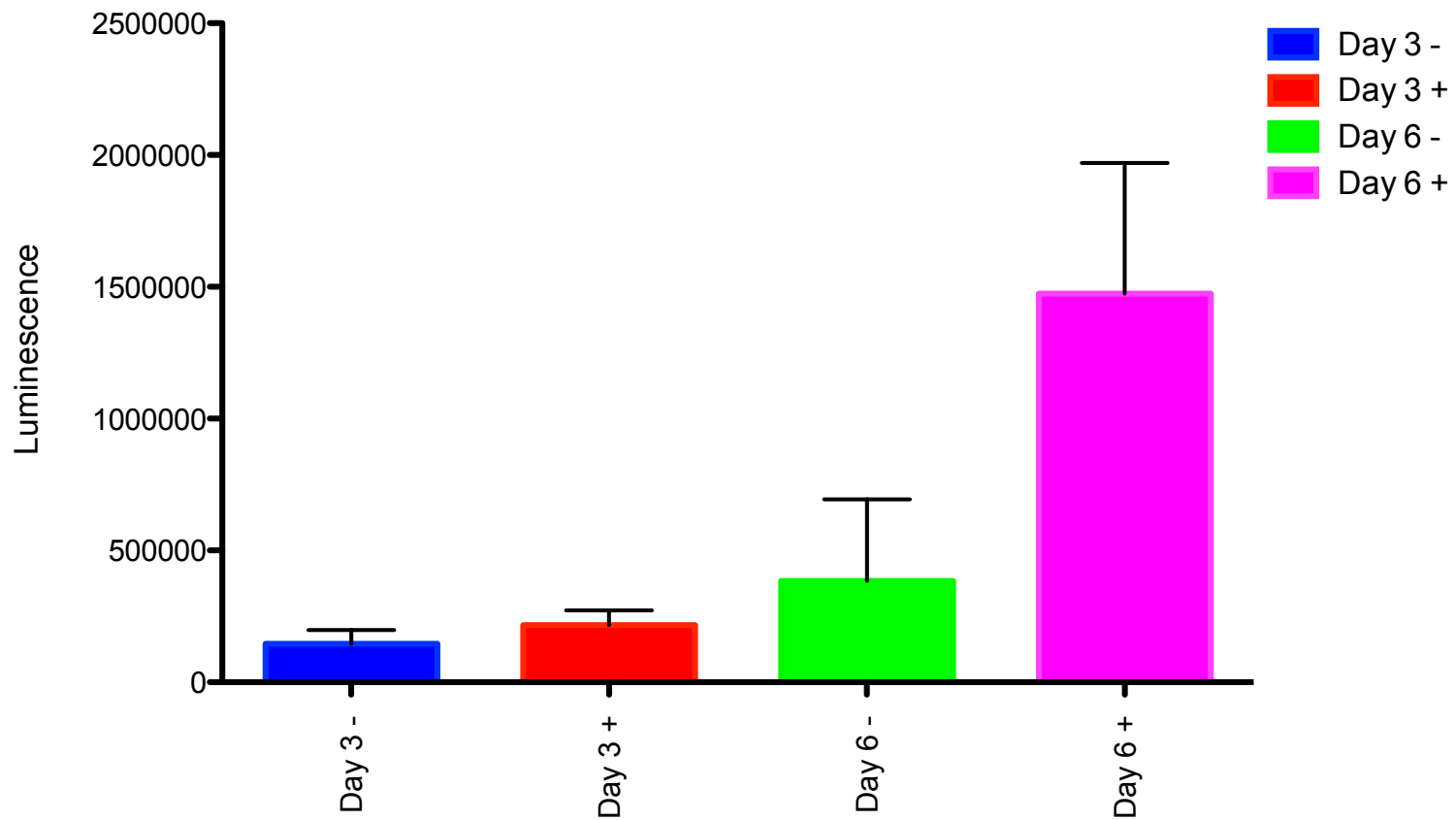
Figure 9. Example of raw data from the screen for BFU-E expansion from replicate analyses of a single 384-well microtiter plate
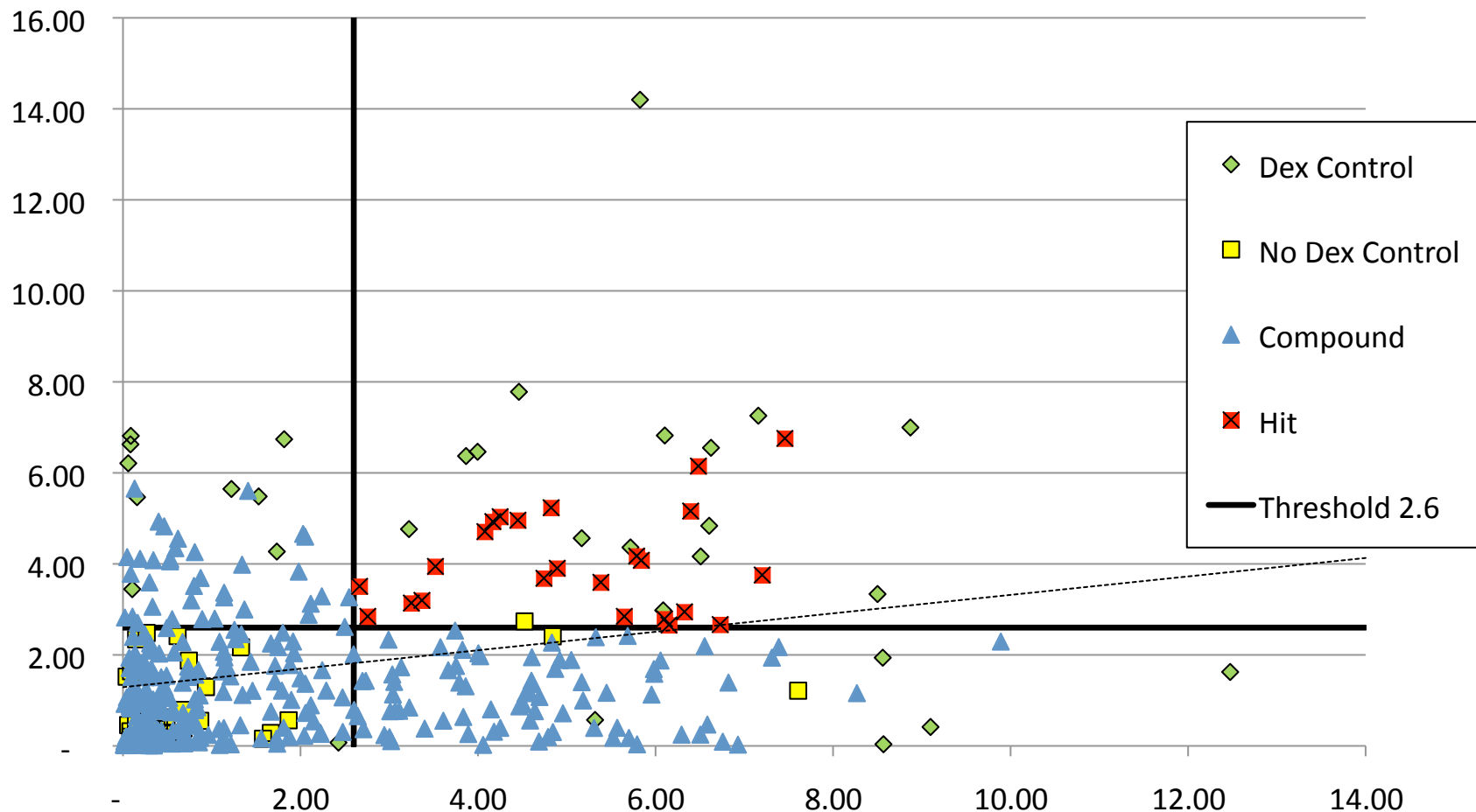
Plotted on each axis are the numbers of cells formed in replicate screenings of the compound in each well of the plate, relative to the average of the control cultures. Details and symbols are described in the text.

Figure 10. Initial rescreening of "hits" that stimulated red cell production in the initial screen
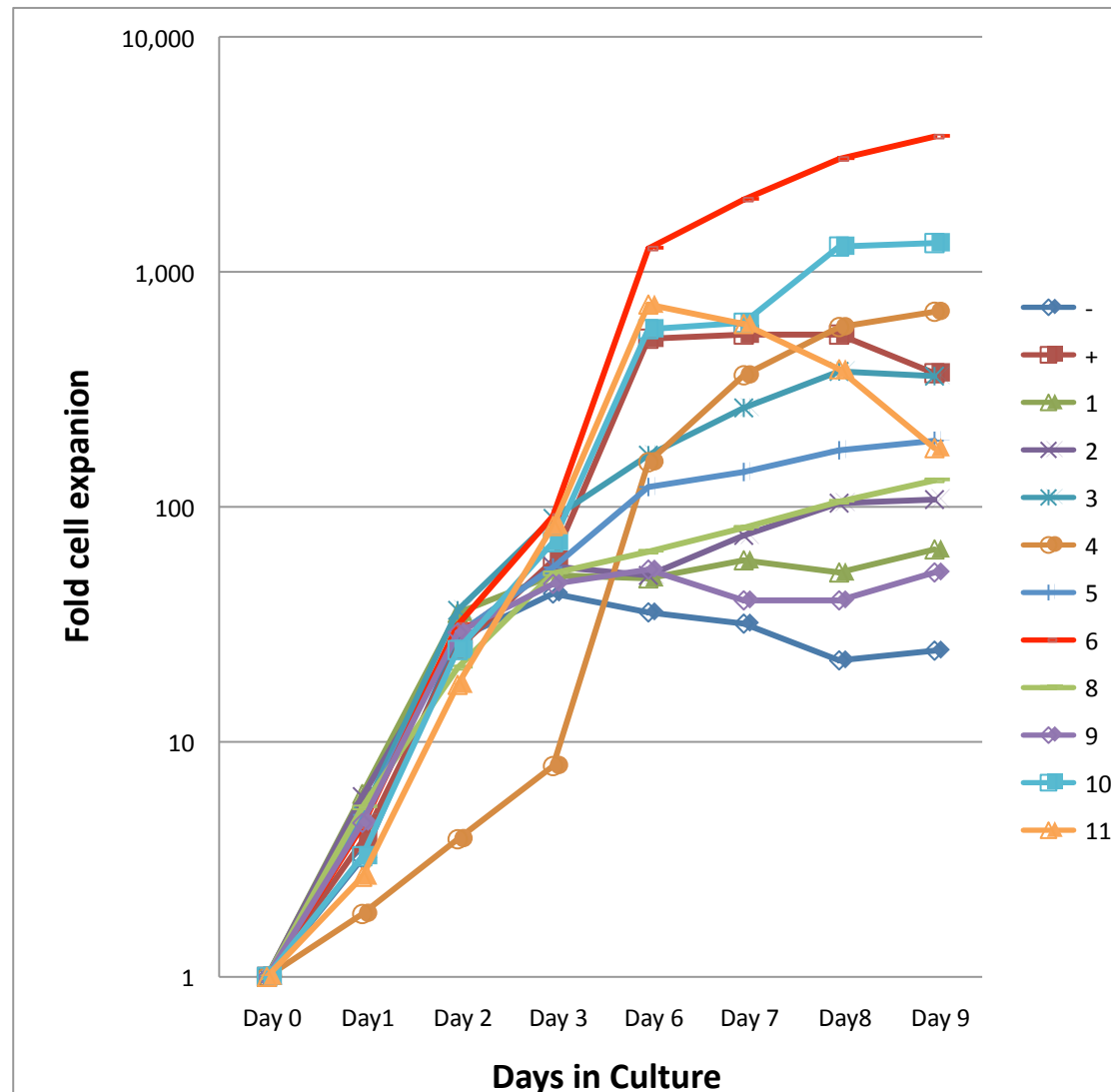
This figure is a plot of the data in Table 1 where a number is assigned to each of the 13 culture conditions
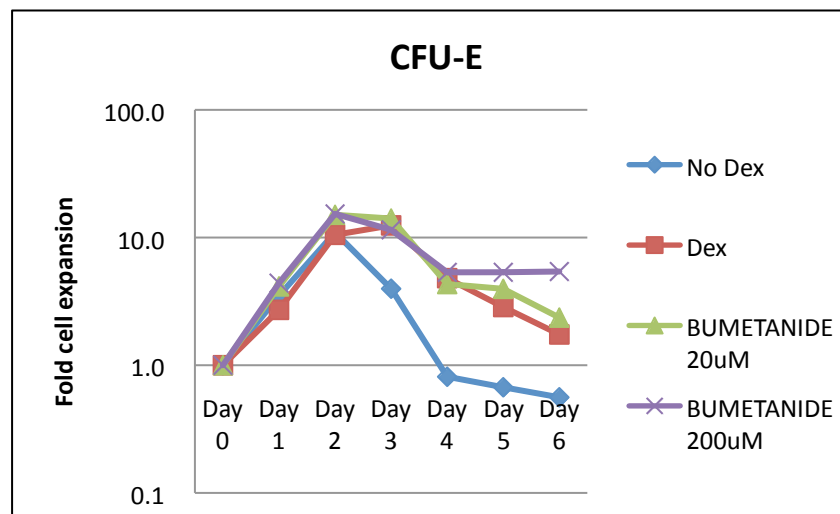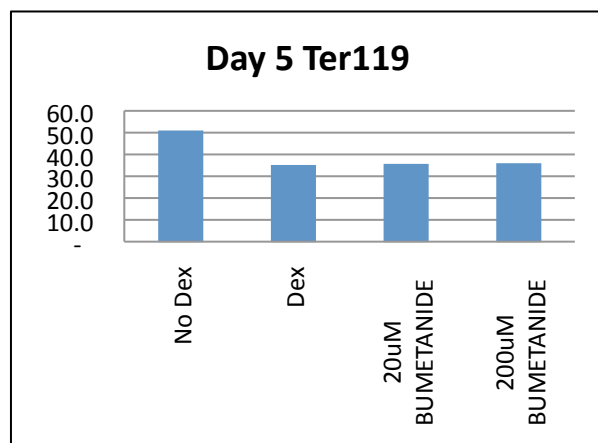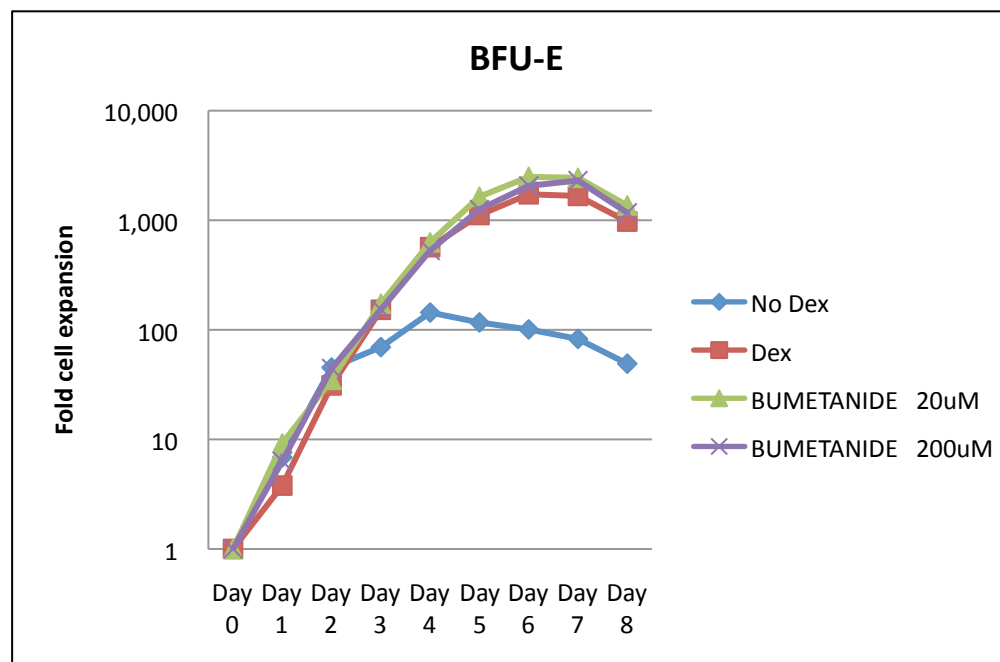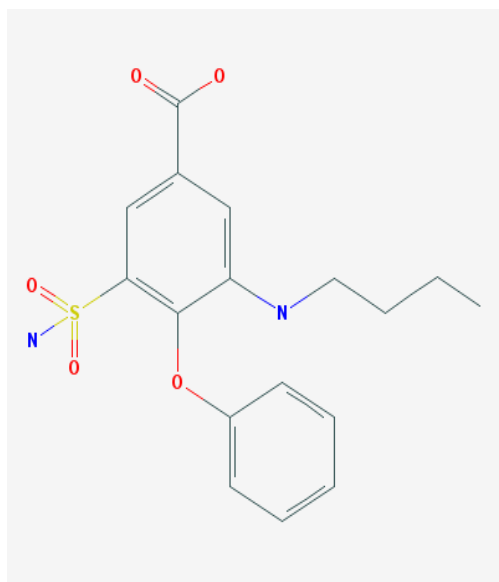
Figure 11 - bumetanide
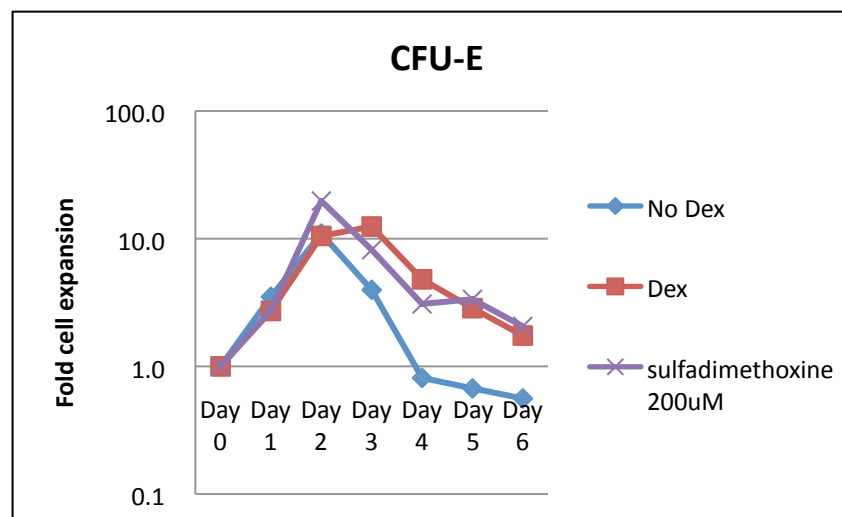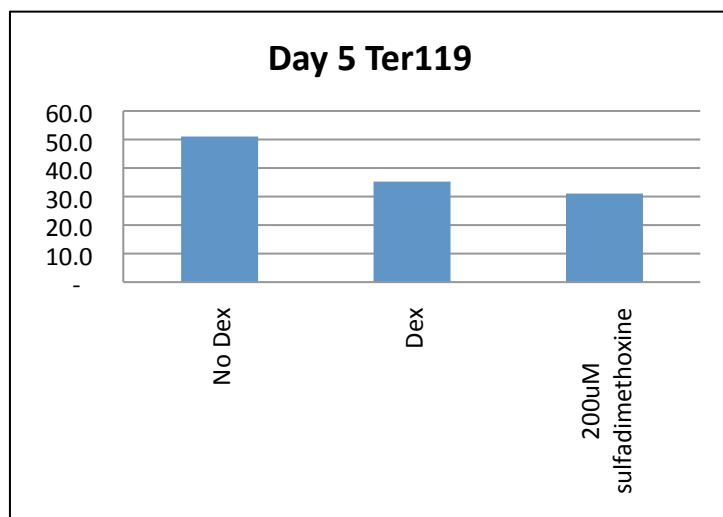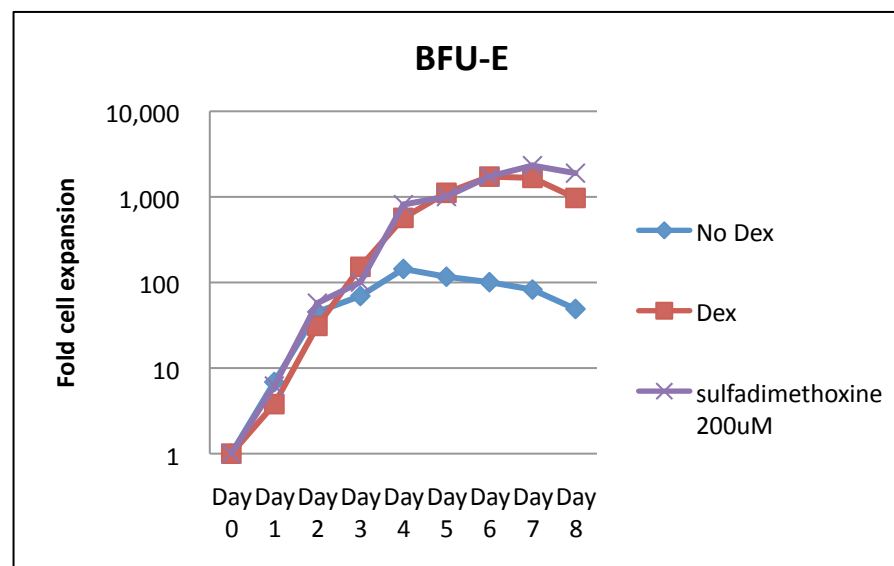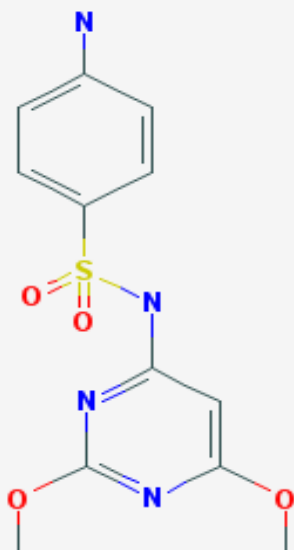
Figure 12 - sulfadimethoxine

BFU-E

Fold cell expansion vs. Day 0 – Day 8

- No Dex
- Dex
- Propylthiouracil 20uM
- Propylthiouracil 200uM

Day 5 Ter119

- No Dex
- Dex
- 20uM Propylthiouracil
- 200uM Propylthiouracil

CFU-E
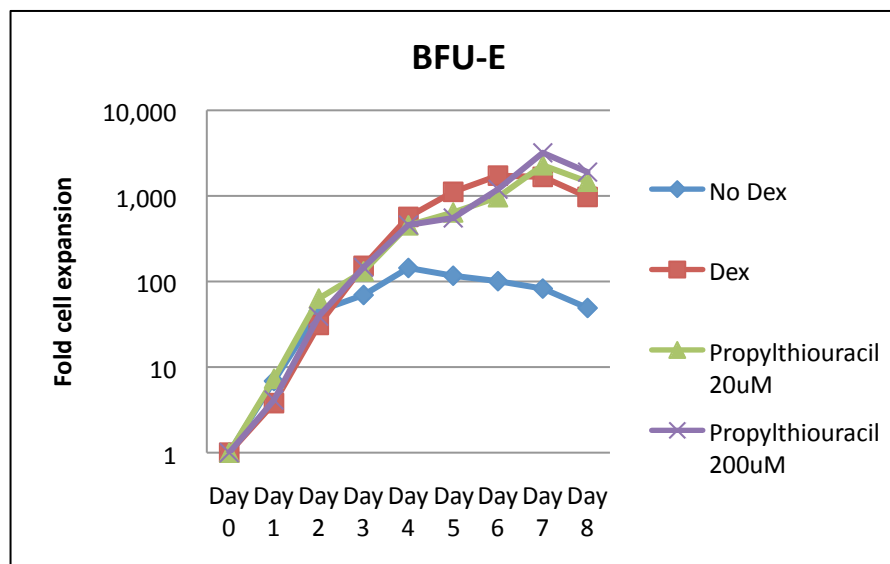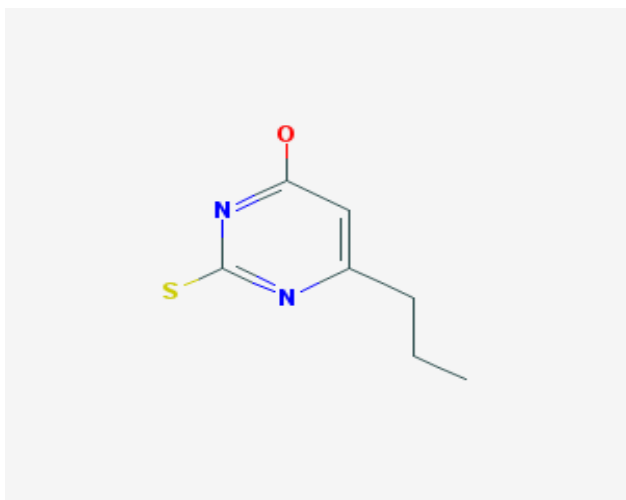
Fold cell expansion vs. Day 0 – Day 6

- No Dex
- Dex
- Propylthiouracil 20uM
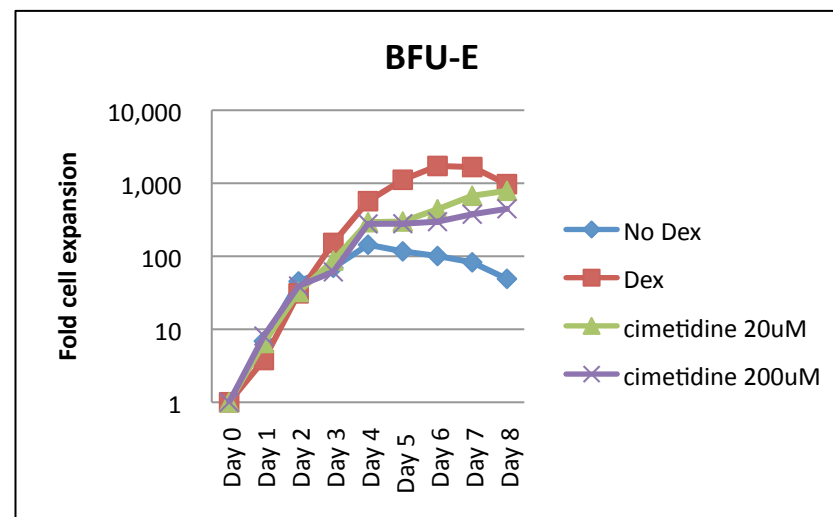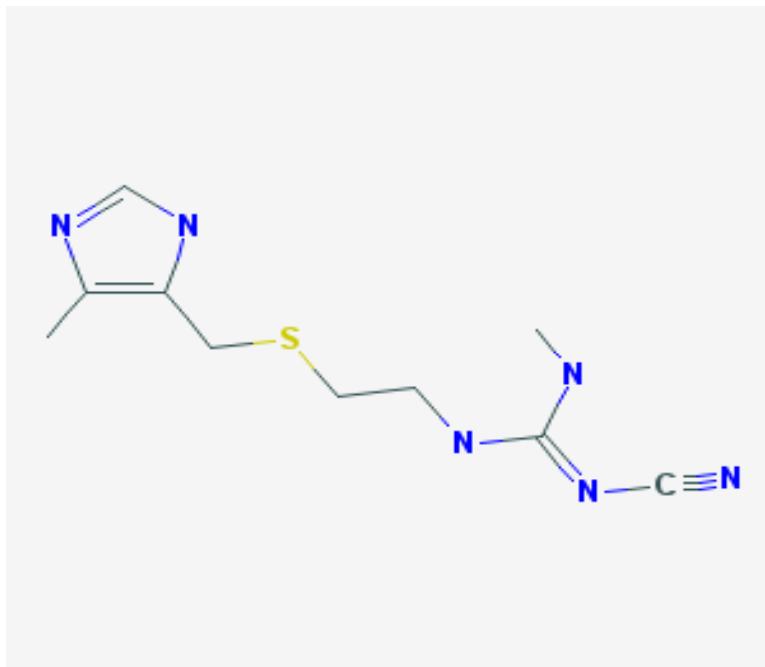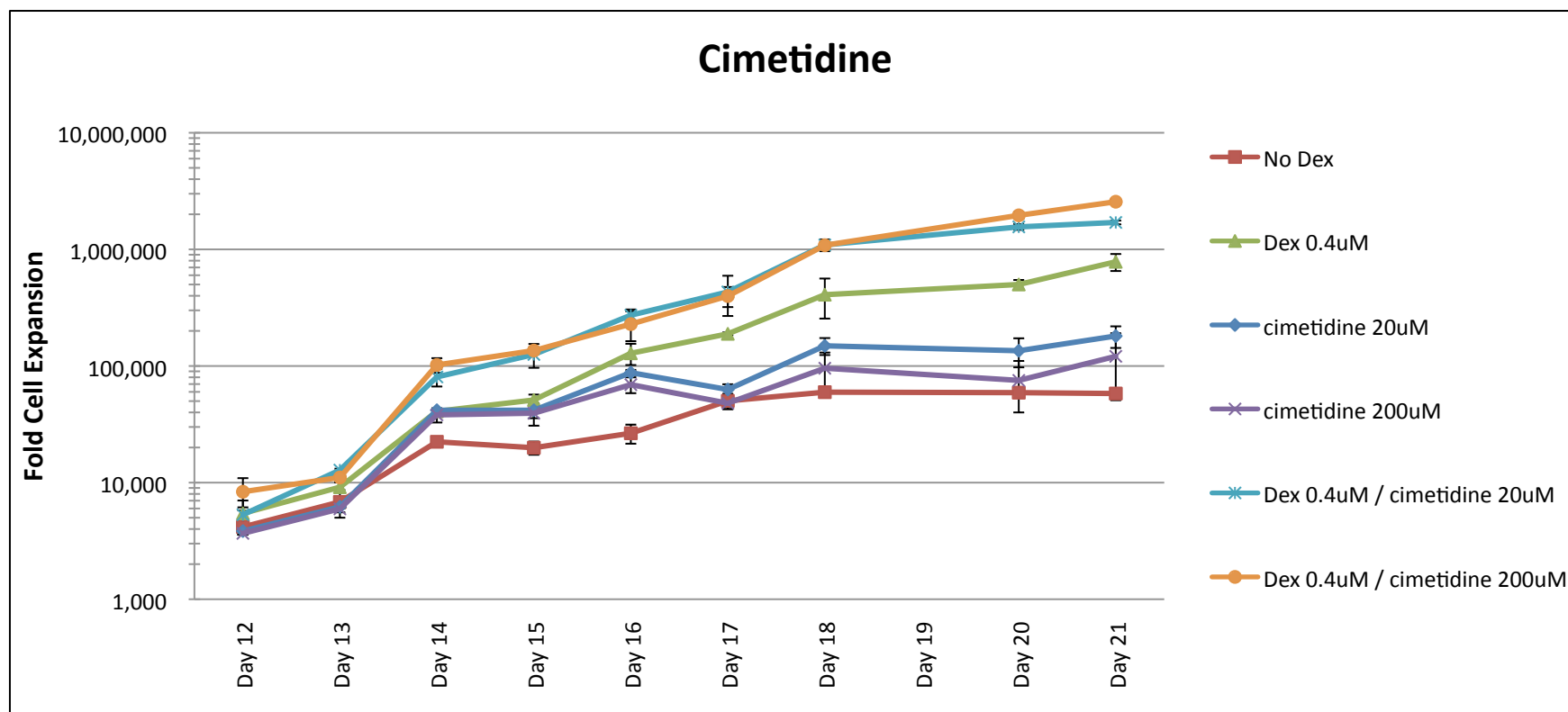- Propylthiouracil 200uM
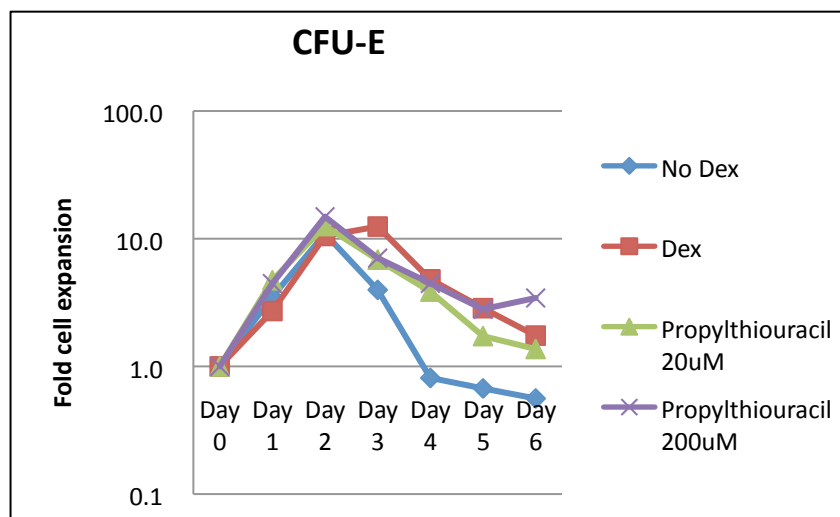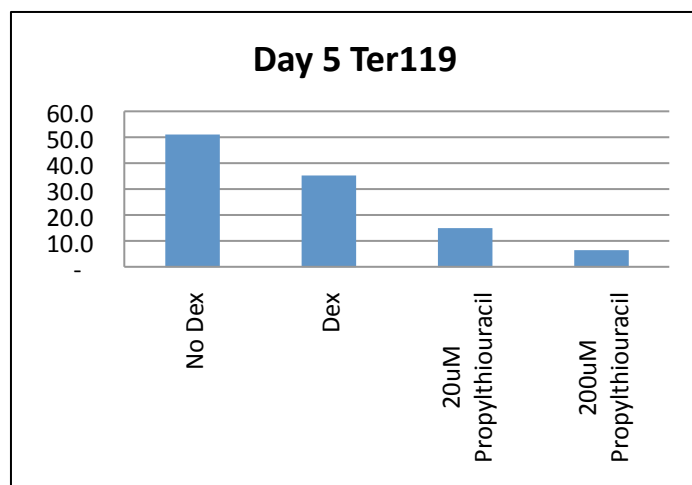
Figure 13 - propylthiouracil
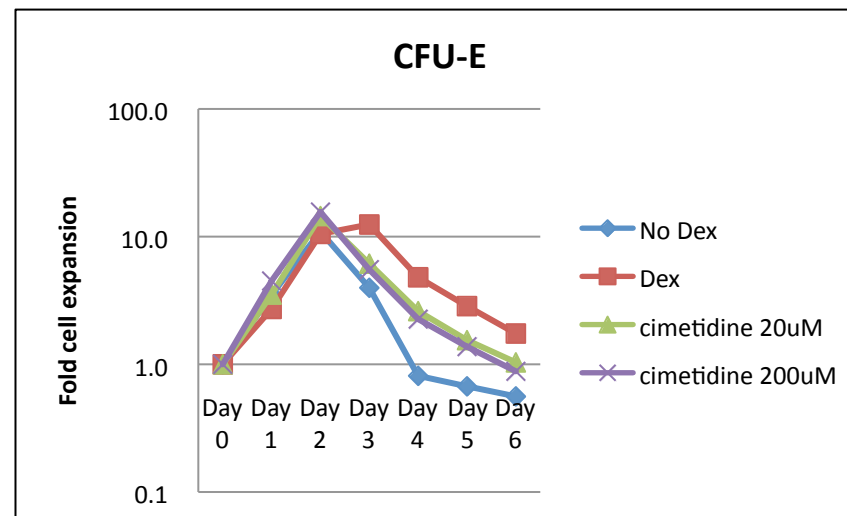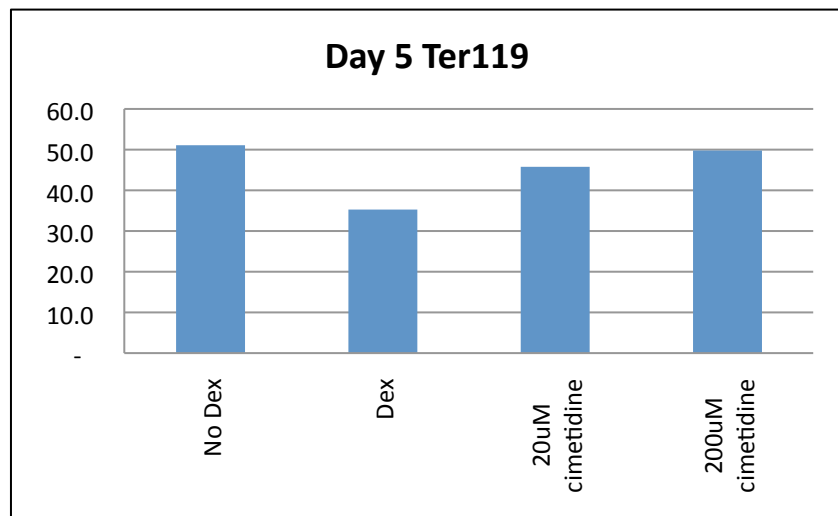
Figure 14 - cimetidine

Figure 15a

Details of the culture conditions are presented in the text. Panel a of this and the subsequent three figures show the total number of cells generated from one plated CD34+ cell; note that the figure plots cell numbers only after Day 12 of culture since all of the cultures with or without drugs did not differ significantly in expansion before that time. Also note that the vertical axis is plotted on a $\log_{10}$ scale.

Figure 15b

Cimetidine CD235a

Legend:
- No Dex
- Dex 0.4uM
- cimetidine 20uM
- cimetidine 200uM
- Dex 0.4uM / cimetidine 20uM
- Dex 0.4uM / cimetidine 200uM

Y-axis: CD235a %
X-axis: Day 9, Day 10, Day 11, Day 12, Day13, Day 14, Day 15, Day 16, Day 17, Day 18, Day 19, Day 20, Day 21
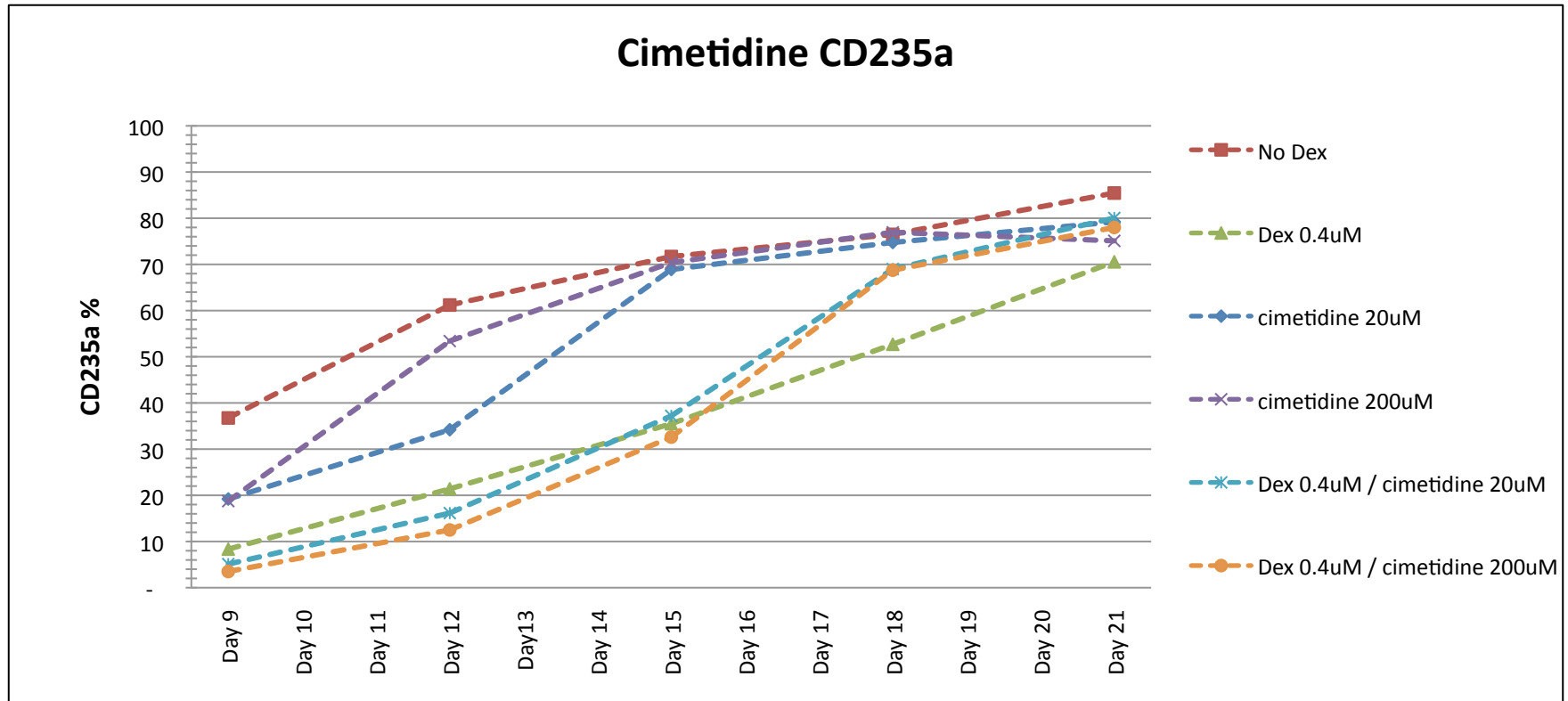
Details of the culture conditions are presented in the text. Panel b of this and the subsequent three figures show the fraction of cells in the cultures that express ther terminal erythroid cell surface protein CD235a, or glycophorin.

Figure 16a

Nizatidine

Legend:
- No Dex
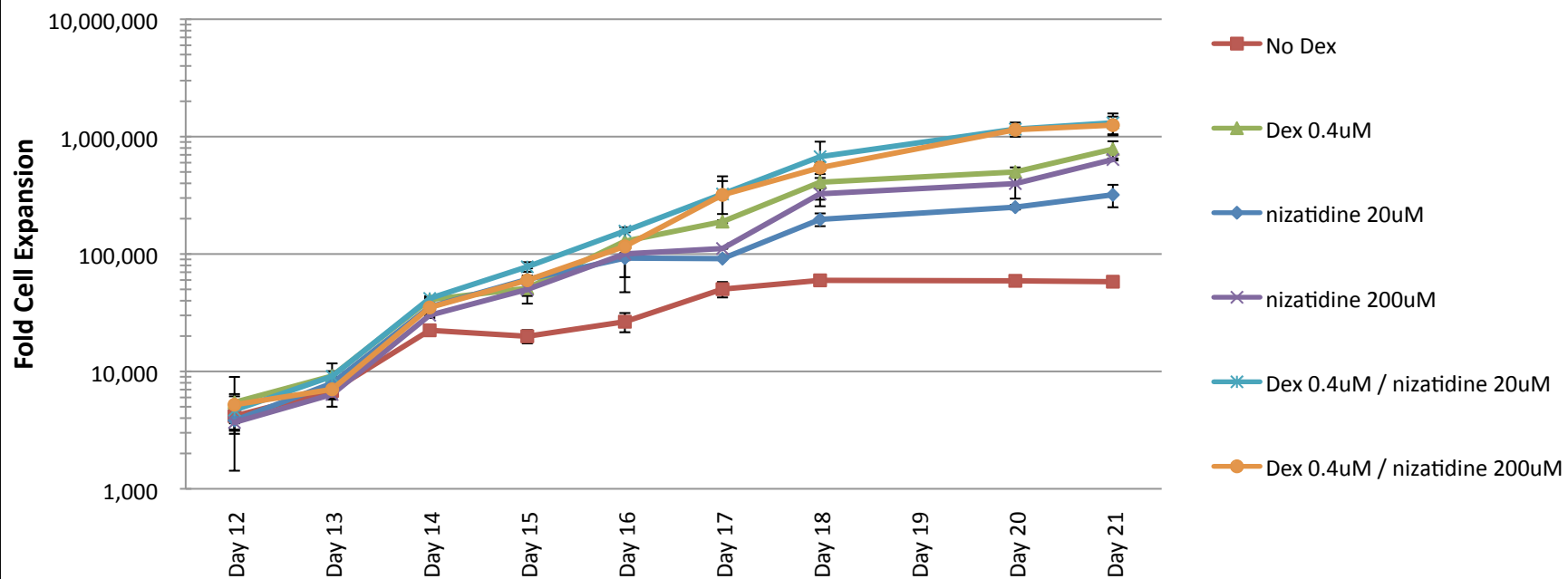- Dex 0.4uM
- nizatidine 20uM
- nizatidine 200uM
- Dex 0.4uM / nizatidine 20uM
- Dex 0.4uM / nizatidine 200uM

Y-axis: Fold Cell Expansion (1,000 to 10,000,000)

X-axis: Day 12, Day 13, Day 14, Day 15, Day 16, Day 17, Day 18, Day 19, Day 20, Day 21

Figure 16b

Figure 16a

Figure 17a

Famotidine

Legend:
- No Dex
- Dex 0.4uM
- famotidine 20uM
- famotidine 200uM
- Dex 0.4uM / famotidine 20uM
- Dex 0.4uM / famotidine 200uM

Y-axis: Fold Cell Expansion (1,000 to 10,000,000)

X-axis: Day 12, Day 13, Day 14, Day 15, Day 16, Day 17, Day 18, Day 19, Day 20, Day 21

Figure 17b

Famotidine CD235a

Figure 18a

Ranitidine

Legend:
- No Dex
- Dex 0.4uM
- ranitidine 20uM
- ranitidine 200uM
- Dex 0.4uM / ranitidine 20uM
- Dex 0.4uM / ranitidine 200uM

Y-axis: Fold Cell Expansion (1,000; 10,000; 100,000; 1,000,000; 10,000,000)

X-axis: Day 12, Day 13, Day 14, Day 15, Day 16, Day 17, Day 18, Day 19, Day 20, Day 21

Figure 18b

Ranitidine CD235a

Legend: No Dex; Dex 0.4uM; ranitidine 20uM; ranitidine 200uM; Dex 0.4uM / ranitidine 20uM; Dex 0.4uM / ranitidine 200uM